STRATEGIES FOR HIGH-THROUGHPUT GENE CLONING AND EXPRESSION

L. J. Dieckman, W. C. Hanly, and F. R. Collart

Biosciences Division
Argonne National Laboratory
Argonne, Illinois 60439

INTRODUCTION

The wealth of data from genomic sequencing projects has led to an increased interest in the development of strategies for high-throughput cloning and expression. The emphasis on the high-throughput component is in part attributable to the initiation of large scale programs such as structural genomics, which mandate the development of automated approaches to facilitate an increased rate of structure determination (1-3). The development of protein chips (4-6) and genomic-scale interaction screens (7, 8) has further stimulated the expansion of high-throughput cloning and expression strategies. However, the experimental approach for development of automated systems for gene cloning and expression is inherently different from classical methods for cloning and expression (9-11). The high-throughput capability of an automated system is achieved at the expense of system flexibility and, as a consequence of this constraint, these strategies usually incur a higher rate of target attrition than more traditional benchtop or low-throughput approaches. The establishment of an

Genetic Engineering, Volume 27, Edited by J. K. Setlow

automated process also requires a more global approach for the evaluation and implementation of cloning and expression protocols (12). Because modifications of established automation methods are expensive and demand significant amounts of time for rewriting and revalidation, protocols must be evaluated at the inception of the program with respect to their compatibility with other method protocols and for feasibility of implementation in an automated setting. We developed a high-throughput cloning and expression strategy from target to validated expression clone that provides a clone resource for the Midwest Center for Structural Genomics (MCSG) (13). This strategy evolved after evaluation of three critical elements common to many high-throughput processes: targets, methods, and screening requirements. Integration of these considerations into a series of methods results in an efficient process that has been scaled to generate thousands of *E. coli* clones. The pipeline incorporates molecular tools that facilitate implementation of parallel processes and allow scaling of the components to meet increasing throughput demands and adapt to changing target characteristics. This chapter will summarize key elements of this process and provide a perspective on high-throughput method development strategies.

AUTOMATION PLATFORM

Commercially available liquid handlers, hardware, and components with standard microplate formats enable integration of high-throughput automation into most basic research departments. The Molecular Biology Robot System at Argonne National Laboratory includes a plate transfer robot (ORCA, Beckman Coulter, Inc., Fullerton, CA) that traverses a 3-m rail system. Adjacent to the rail are a number of stations (such as pipetting workstations, a plate washer, shaker, heatblock, barcode reader, incubators, etc.) that perform the equivalent of standard laboratory molecular biology manipulations during an automation procedure. The liquid handling stations include Beckman Coulter Biomek 2000 and Multimek workstations. The Biomek 2000 workstation incorporates a filtration station for purification of plasmids and amplified fragment DNA, a gripper device to allow for movement of labware, and thermal reservoirs to allow for heating/cooling of microwell plates. The system is controlled *via* the SAMI NT software package that provides a graphical interface for the development, scheduling, and implementation of methods on the system. For most applications, the graphical interface enables benchtop-trained scientists to design and implement methods without the need for a dedicated automation specialist.

HIGH-THROUGHPUT STRATEGY

Targets

The cloning and expression pipeline producing clones for the MCSG is one of the Protein Structure Initiative pilot centers funded by the National Institute of General Medical Sciences at the National Institutes of Health (1). The structural genomic target set of the MCSG represents mostly microbial targets characterized as cytoplasmic proteins. The characteristics of the target set

suggest *Escherichia coli* as a logical first choice of expression system in view of its demonstrated utility for expression of microbial proteins (14, 15). This system represents an efficient approach to produce proteins quickly, in large amounts, and in a cost efficient manner (16, 17) and is the standard platform used by a number of structural genomics centers as the primary protein production platform (12, 18-20).

Although prokaryotic expression systems have many advantages for both small- and large-scale protein expression, they have some limitations due to the inability of a prokaryotic system to produce proteins in as complex a manner as a eukaryotic cell (21-23). However, a number of laboratories (9, 19) have implemented large-scale platforms based in whole or in part on the *E. coli* expression system and have developed high-throughput methods for successful expression of eukaryotic proteins. These platforms typically employ multiple strategies involving both genetic design and protein expression cassettes in *E. coli* to maximize the generation of soluble proteins or protein domains for downstream analysis (19, 22, 24). A common approach is to use a bacterial expression system as an initial platform and screen for the production of soluble proteins. Targets that fail in the initial round can then be routed through salvage pathways that utilize alternative expression strategies. This tiered strategy leverages the efficient and cost-effective high-throughput processes that are already available for the production of proteins in *E. coli* before proceeding to more expensive and time intensive alternative approaches.

The characteristics of the target set extend beyond the choice of expression system(s) and impact the core methods and design of the protein production pipeline. Targets for the structural genomics pilot centers are selected by a criterion of less than 30% sequence identity to sequences in the Protein Data Bank. This constraint results in a target group containing large numbers of uncharacterized and hypothetical proteins that represent a challenge for expression in a soluble form. For the structural genomics centers, the metric for success at the expression level is the production of a clone expressing soluble protein at a level that enables purification of a sufficient amount of protein for crystallization trials. Large-scale expression studies addressing cytoplasmic targets suggest a capability to express approximately 30-50% of targets in a soluble form (13, 25). However, it can be anticipated that the process for the generation of clones expressing soluble protein at the level required for crystallization screening will become more difficult as the "low-hanging fruit" component of the target set is depleted (26, 27). These considerations have led to the development and implementation of high-throughput screening strategies for identification of clones expressing soluble protein that allow for higher overall throughput, reduced costs, and significant improvement of the efficiency of the production process.

High-Throughput Methods

The cloning and expression strategy for protein production represents a critical decision element of the high-throughput production process. This decision impacts most elements of the process, including the front-end bioinformatics, vectors, and targets, and the selection of methods appropriate for implementation in

an automated environment. The cloning strategy also influences the outcome metrics of cost, efficiency. and throughput. For many of the structural genomic pilot centers, the need to insure a constant supply of validated expression clones for crystallization trials has led to the implementation of parallel cloning strategies to ameliorate some attrition due to the production of low solubility or insoluble proteins. This type of parallel strategy relies on a universal cloning site approach for cloning and expression of targets in multiple vectors and from many sources. Although several universal cloning site systems are presently available, we selected the Ligation Independent Cloning (LIC) method (28, 29) for implementation into our high-throughput protein production pipeline. The selection of this method was based on system characteristics that facilitated implementation of the process in an automated environment as well as global considerations such as cost and efficiency. Some of the major attributes of this system that impacted the decision to select the LIC method as a core cloning strategy are summarized below:

- Our analysis of the heterogeneous character (a large component of hypothetical and uncharacterized open reading frames, ORFs), of the targets selected for structural genomics indicated that multiple expression systems would be advantageous to achieve a representative array of clones that expressed a soluble protein product. The capability to utilize multiple vectors and hosts in an automated process to generate and screen expression clones is an essential component to increase the target success rate for many high-throughput protein expression strategies. In the LIC approach, universal cloning sites can be incorporated at the primer design stage, enabling a general cloning approach to most of the selected targets (30). This characteristic enables implementation of parallel methods that utilize multiple vectors. Although a variety of vectors are available for the *E. coli* hosts, the selection of an optimal vector is dependent on programmatic goals and automation requirements (9, 31, 32). Our experience and that of others, however, indicates it is often necessary to design an array of compatible vector systems to provide for flexibility of expression with different fusion tags and protease cleavage sites (33, 34).
- The LIC approach employs a directional cloning method that simplifies robotic implementation by reducing the number of processes necessary to generate a validated clone. The directional nature allows for direct expression screening and facilitates the development of efficient screening methods for soluble expression products.
- The LIC cloning method does not involve restriction enzymes. This consideration eliminates restriction site screening as a component of the target selection process and enables a single cloning protocol to be applied to all targets.
- A characteristic of the LIC cloning method and some other universal cloning systems is the relatively simple methodologies for clone preparation. In automated environments, reducing the method complexity facilitates implementation and eliminates the occurrence of processing errors. The LIC approach is easily adaptable to an automation system inasmuch as the method requires only one enzymatic step that occurs at room temperature. Furthermore, the time required for processing of plates is minimal and the reagents used in the process are stable at the cold block temperatures that are maintained during transport on the automation system.

- A critical step in the cloning process is the combination of the target DNA with the vector. In the LIC method, this occurs *via* an annealing reaction conducted at room temperature. Our studies of various fragment to vector ratios (Figure 1) indicate a wide tolerance for variation in the amount of target DNA fragment on the annealing reaction. This latitude eliminates the need for normalization of fragment concentrations prior to annealing, thus conserving time and simplifying the process for implementation of the method as an automated process.
- The LIC method is highly efficient and cost effective. In a large-scale study of more than 880 targets from *Bacillus subtilis*, our analysis of individual LIC expression clones produced in the microwell plates indicated an overall expression efficiency of approximately 70% for all targets and vectors (35). Manual analysis of multiple clones for each target (four clones from each target were analyzed by denaturing gel electrophoresis) demonstrated expression of the target protein in greater than 75% of screened clones (13). This high expression efficiency provides several options for implementing expression screening procedures. One option implemented at the MCSG includes screening of nonclonal plasmid stocks for expression and solubility prior to clone isolation and storage. This process is desirable when the expected rate of target attrition is likely to be high to avoid time intensive cloning procedures for nonproductive clones. In any case, the high expression efficiency associated with the LIC method minimizes the amount of downstream effort required for the selection and validation of individual expression clones.

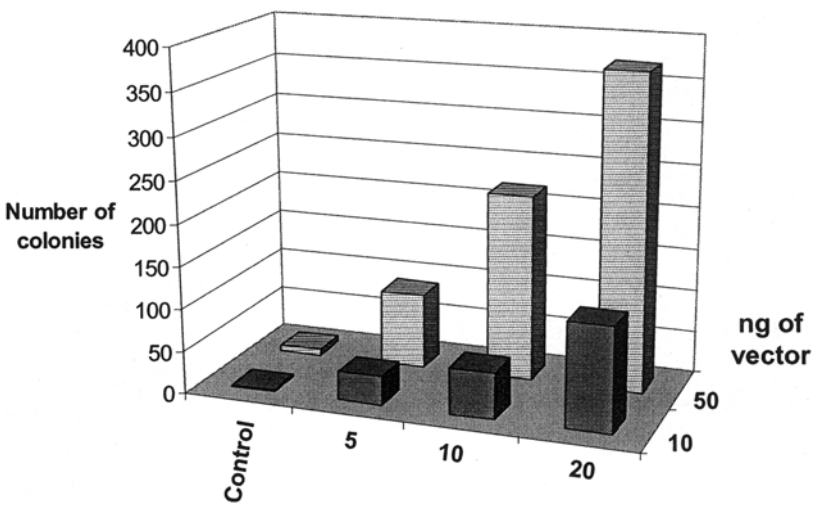

Figure 1. Dependence of colony formation at various fragment to vector ratios. The indicated amounts of LIC fragment and vector were annealed for 10 minutes prior to transformation. Control samples contained vector but no added LIC fragment.

The LIC cloning method was selected as a core strategy because these characteristics matched the requirements of the structural genomics program. Specific advantages include efficiency, cost, and ability to implement parallel approaches for different vectors. A disadvantage of the LIC approach is the reliance on the polymerase chain reaction (PCR) fragment as the cloning entry point. Due to the high attrition for structural genomics targets, individual targets are not cloned until a target is tested for soluble protein expression. Although this represents a cost-effective method for clone production, modifications of the LIC approach (36) as well as alternative approaches (20, 37) have been successfully implemented in the Protein Structure Initiative pilot centers.

Tag Detection Screening for Soluble Proteins

For the MCSG and many other high-throughput protein production centers, the measure of success for high-throughput cloning and expression component is the generation of a clone expressing a soluble protein product. The historical success rate for production of clones expressing soluble proteins (<50%) and the uncharacterized nature of the target group, suggested that implementation of a microwell plate–based screening method could reduce the amount of time spent on expression and solubility validation of nonproductive clones. This realization has led to the development of a number of high-throughput screening strategies for soluble proteins (38, 39) with approaches ranging from tag detection (40) to genetic endpoints (41, 42). For a high-throughput screening method to be effective it must be rapid and reproducible, and it must be able to predict which clones will be able to produce soluble proteins in culture. The day-to-day variations must be sufficiently low so that the test does not need to be repeated for reliable results. The purpose of the high-throughput screening is not to produce proteins of immediate use to the investigator but to indicate which clones will express soluble proteins for large-scale expression and further functional or structural studies. When working with large numbers of clones at a time, as is frequently done in high-throughput protein production, it is useful to evaluate carefully as many clones as possible before conducting manual and/or large-scale purification. The ultimate goal of the screening process is to identify a population of clones that are likely to give a high success rate in large scale protein purification (43-45).

We developed an expression screen for production of soluble protein that uses a tag detection strategy to screen for production of protein containing the 6x histidine component of the fusion tag (33). India HIS Probe-HRP (Pierce Chemical, Rockford, IL), a nickel activated horseradish peroxidase, is used for detection of the 6x histidine component of the fusion tag. The assay was developed using standard 96-well plates containing 88 target clones and 8 control wells. The four-day procedure enables processing of up to eight plates of clones with standard liquid handlers (13, 35). On the first day of the process, plasmid DNA is transformed into chemically competent BL21 cells, which are cultured overnight. The next day, the overnight cultures are diluted and incubated at 37°C (to log phase absorbance) before induction with isopropyl thiogalactoside. After two hours of further incubation, cells are lysed by a two-step

process with a sodium phosphate–buffered solution containing lysozyme, benzonase nuclease, and a 25% detergent solution. The tag detection assay is run on the third day but can be performed on the second day, provided sufficient time has elapsed for adsorption of the expressed proteins to the Immulon 4HBX plates.

The characteristics of the tag detection screen were assessed by intensive screening of 2 of the 10 plates of targets from a MCSG *Bacillus subtilis* genome screen (35). These plates, designated Bsub04 and Bsub08, were used for all of the following experiments. Reproducibility of the tag detection assay was assessed by conducting a screen on samples generated from the same bacterial growth culture and performing the assay on successive days. Differences were calculated by subtracting each sample's rank on day 1 from day 2 of the tag detection assay (the ranking procedure is described in the legend to Figure 2). An average deviation of approximately five positions was observed in the ranking of all 88 samples (Figure 2), suggesting this approach has value as a preliminary screen for detection of the fusion tag. The overall reproducibility of the experiment from bacterial transformation to the tag detection assay was assessed by averaging all ranking data for all of the assays from each plate (Bsub04 and Bsub08). We observed an overall standard deviation of approximately 12 rank positions averaged over all assays and plates (Table 1). These variations are attributable in part to the multiple pipetting and plate washing procedures on the robotic system, which occur over the three-day process. Variations in bacterial culture growth were also observed (not shown), and most likely contributed to the ranking differences found in Table 1.

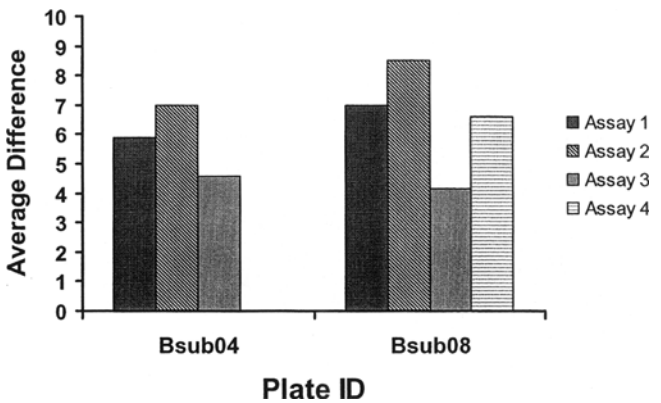

Figure 2. Day-to-day variation of target ranking in tag-detection screen with the same induced growth samples. The average differences and standard deviations were calculated from the absolute values of all 88 samples. The 88 plate samples in individual plates were ranked such that the well with the highest absorbance at 450 nm received a score of 1, whereas the well with the lowest absorbance at 450 nm received a score of 88. Ranking data were pooled for nine assay plates representing four different bacterial growth dates for Bsub04 clones and 11 assay plates representing five different bacterial growth dates for Bsub08 clones (Table 1). Average ranking score and standard deviations were calculated from the absolute values of all 88 samples for the nine assay plates.

Table 1. Overall reproducibility of tag detection assay.

	Assays	Range	Average Std. Deviation
Bsub04	9	1.00-25.56	11.01
Bsub08	11	2.73-27.38	13.40

The ability of the tag detection assay to predict expression of soluble proteins was evaluated by comparison of the results from the tag detection assay to those obtained from SDS-PAGE gel analysis of the soluble fractions. The data were pooled for all tag detection assays and each target in the plate assigned to one of four ranking groups (legend to Figure 2). Each of the groups contained 22 targets sorted so that the highest ranked group contained the targets with the highest scores 1-22 in the tag detection assay. (A score of 1 indicated the highest amount of color development on the assay plate.) For the Bsub08 plate, a total of 58 soluble positive expression clones were found. Twenty-one positive expression clones were found in the highest ranking group, with 20, 12, and 5 positive expression clones found in the remaining groups as ranked from highest to lowest scores (Figure 3). A similar trend was observed in the Bsub04 plate in that the targets from the tag detection assay with the highest scores were most likely to be associated with identification of a soluble protein band after SDS-PAGE analysis (Figure 3).

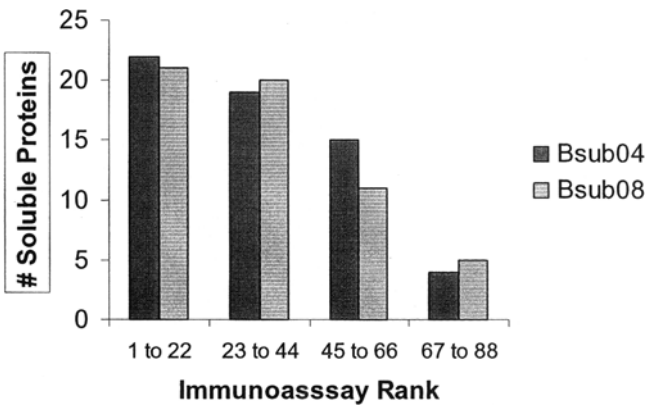

Figure 3. Summary of solubility *versus* relative rank as determined by the tag detection assay. For Bsub04, ranking data were pooled for nine assay plates representing four different bacterial growth dates. For Bsub08, ranking data were pooled for 11 assay plates representing five different bacterial growth dates. For solubility data, SDS-PAGE gels were evaluated by visually analyzing and scoring the gel according to the protein band found on the gel. If no protein band was apparent in the correct molecular weight region, the protein was given a score of 0. Scores of 1 and 2 were given to bands that were present indicating low and high solubility, respectively. All clones receiving a score over 0 were considered positive expression clones.

These data show that a positive expression clone can be predicted from assay rank. The highest-ranked 25%, or highest 22 from the Bsub04 assay ranking data, gave 22 soluble expression clones as analyzed by SDS-PAGE. Twenty-one out of 22 of these proteins received the highest solubility score of 2 (see legend to Fig. 3 for description of solubility level assignments). In the lowest-ranked 25% of the plate by assay, four soluble clones were found; three of these four received solubility scores of 1. The highest-ranked 25%, or highest 22 from the Bsub08 assay ranking data, gave 21 soluble expression clones as analyzed by SDS-PAGE. Sixteen out of 22 of these proteins received the highest solubility score. In the lowest-ranked 25% of the assay plate, five soluble clones were found; three of these received solubility scores of 1.

In order to test the ability of the assay to evaluate the soluble expression clones in the context of a single screen (the norm for an HTP production run), we analyzed tag detection results from a single plate and set of SDS-PAGE gels. For the Bsub04 plate, the highest ranked 22 clones on the assay plate gave 22 soluble expression clones as analyzed by SDS-PAGE (Figure 4). In the lowest-ranked 25% of the plate, one soluble expression clone was found by SDS-PAGE. The highest-ranked 25%, or highest 22 clones from the Bsub08 plate, gave 21 soluble expression clones as analyzed by SDS-PAGE. In the lowest-ranked 25% of the plate by tag detection assay, three soluble clones were found. These data show a positive expression clone can be predicted from the tag detection assay rank and thus eliminate downstream screening cost and time.

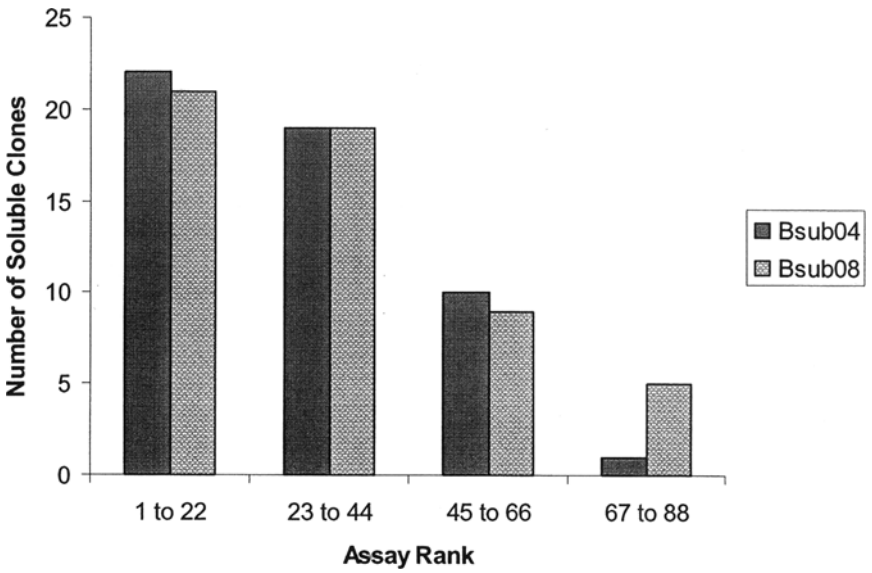

Figure 4. Prediction capability of the tag detection screen from a single growth plate. A single induced bacterial culture plate was used to generate two tag detection assay plates and one set of SDS-PAGE gels. Ranking data for the two days were averaged and plotted against the solubility data.

Although the data from the tag detection assay correlated well with the prediction of soluble clones, there were a few proteins in which the solubility rankings and SDS-PAGE results did not correlate. Several of the proteins that scored high in the tag detection assay ranking did not produce a soluble clone on SDS-PAGE. A protein that is cleaved or incompletely transcribed would produce a small peptide that may not be detectable on SDS-PAGE and yet give a strong signal on the tag detection assay. In addition, several proteins that received low rankings in the tag detection assay produced soluble proteins when screened by SDS-PAGE. These may represent proteins in which the his tag is buried or unavailable to the his-probe; they would not be detected on the tag detection screen even if they were expressed and soluble.

SUMMARY

High-throughput approaches for gene cloning and expression require the development of new, nonstandard tools for use by molecular biologists and biochemists. We have developed and implemented a series of methods that enable the production of expression constructs in 96-well plate format. A screening process is described that facilitates the identification of bacterial clones expressing soluble protein. Application of the solubility screen then provides a plate map that identifies the location of wells containing clones producing soluble proteins. A series of semi-automated methods can then be applied for validation of solubility and production of freezer stocks for the protein production group. This process provides an 80% success rate for the identification of clones producing soluble protein and results in a significant decrease in the level of effort required for the labor-intensive components of validation and preparation of freezer stocks. This process is customized for large-scale structural genomics programs that rely on the production of large amounts of soluble proteins for crystallization trials.

ACKNOWLEDGMENTS

The authors thank Andrzej Joachimiak and Fred Stevens for their support of the high-throughput cloning and expression efforts. This work was supported by National Institutes of Health Grant GM62414-01, A. Joachimiak, PI, and by the U.S. Department of Energy, Office of Health and Environmental Research, under contract W-31-109-Eng-38.

REFERENCES

1 Norvell, J.C. and Machalek, A.Z. (2000) Nat. Struct. Biol. 7 Suppl., 931.
2 Schmid, M.B. (2004) Nat. Rev. Microbiol. 2(9), 739-746.
3 Zhang, C. and Kim, S.H. (2003) Curr. Opin. Chem. Biol. 7(1), 28-32.
4 Jung, G.Y. and Stephanopoulos, G. (2004) Science 304, 428-431.
5 Lee, Y.S. and Mrksich, M. (2002) Trends Biotechnol. 20(12 Suppl.), S14-S18.
6 Phelan, M.L. and Nock, S. (2003) Proteomics 3(11), 2123-2134.

7 Lehner, B. and Fraser, A.G. (2004) Genome Biol. 5(9), R63.
8 Terradot, L. Durnell, N., Li, M., Li, J., Ory, J., Labigne, A., Legrain, P., Colland, F. and Waksman, G. (2004) Mol. Cell. Proteomics 3(8), 809-819.
9 Chambers, S.P. (2002) Drug Discov. Today 7(14), 759-765.
10 Endo, Y. and Sawasaki, T. (2003) Biotechnol. Adv. 21(8), 695-713.
11 Sarafanov, A. and Saenko, E. (2004) Anal. Biochem. 328(1), 98-100.
12 Lesley, S.A. (2001) Protein Expr. Purif. 22(2), 159-164.
13 Dieckman, L., Gu, M., Stols, L., Donnelly, M.I. and Collart, F.R. (2002) Protein Expr. Purif. 25(1), 1-7.
14 Savchenko, A., Yee, A., Khachatryan, A., Skarina, T., Evdokimova, E., Pavlova, M., Semesi, A., Northey, J., Beasley, S., Lan, N., Das, R., Gerstein, M., Arrowmith, C.H. and Edwards, A.M. (2003) Proteins 50(3), 392-399.
15 Yee, A., Pardee, K., Christendat, D., Savchenko, A., Edwards, A.M. and Arrowsmith, C.H.. (2003) Acc. Chem. Res. 36(3), 183-189.
16 Baneyx, F. (1999) Curr. Opin. Biotechnol. 10(5), 411-421.
17 Baneyx, F. and Palumbo, J.L. (2003) Methods Mol. Biol. 205, 171-197.
18 Chance, M.R., Fiser, A., Sali, A., Pieper, U., Eswar, N., Xu, G., Fajardo, J.E., Radhakannan, T. and Marinkovic, N. (2004) Genome Res. 14(10B), 2145-2154.
19 Folkers, G.E., van Buuren, B.N. and Kaptein, R. (2004) J. Struct. Funct. Genomics 5(1-2), 119-131.
20 Yokoyama, S. (2003) Curr. Opin. Chem. Biol. 7(1), 39-43.
21 Baneyx, F. (1999) Curr. Opin. Biotechnol. 10(5), 411-421.
22 Chambers, S.P., Austen, D.A., Fulghum, J.R. and Kim, W.M. (2004) Protein Expr. Purif. 36(1), 40-47.
23 Laage, R. and Langosch, D. (2001) Traffic 2(2), 99-104.
24 Uchimura, Y., Nakamura, M., Sugasawa, K., Nakao, M. and Saitoh, H. (2004) Anal. Biochem. 331(1), 204-206.
25 DiDonato, M., Deacon, A.M., Klock, H.E., McMullan, D. and Lesley, S.A. (2004) J. Struct. Funct. Genomics 5(1-2), 133-146.
26 Janda, I., Devedjiev, Y., Cooper, D., Chruszcz, M., Derewenda, U., Gabrys, A., Minor, W., Joachimiak, A. and Derewenda, Z.S. (2004) Acta Crystallogr. D Biol. Crystallogr. 60(Pt 6), 1101-1107.
27 Lundstrom, K. (2004) Comb. Chem. High Throughput. Screen. 7(5), 431-439.
28 Haun, R.S., Serventi, I.M. and Moss, J. (1992) Biotechniques 13(4), 515-518.
29 Aslanidis, C. and de Jong, P.J. (1990) Nucl. Acids Res. 18(20), 6069-6074.
30 Yoon, J.R., Laible, P.D., Gu, M., Scott, H.N. and Collart, F.R. (2002) Biotechniques 33(6), 1328-1333.
31 di Guan, C., Li, P., Riggs, P.D. and Inouye, H. (1988) Gene 67(1), 21-30.
32 Routzahn, K.M. and Waugh, D.S. (2002) J. Struct. Funct. Genomics 2(2), 83-92.
33 Stols, L., Gu, M., Dieckman, L., Raffen, R., Collart, F.R. and Donnelly, M.I. (2002) Protein Expr. Purif. 25(1), 8-15.

34 Woestenenk, E.A., Hammarstrom, M., van den Berg, S., Hard, T. and Berglund, H. (2004) J. Struct. Funct. Genomics 5(3), 217-229.
35 Moy, S., Dieckman, L., Schiffer, M., Maltsev, N., Yu, G.X. and Collart, F.R. (2004) J. Struct. Funct. Genomics 5(1-2), 103-109.
36 Nguyen, H., Martinez, B., Oganesyan, N. and Kim, R. (2004) J. Struct. Funct. Genomics 5(1-2), 23-27.
37 Finley, J.B., Qiu, S.H., Luan, C.H. and Luo, M. (2004) Protein Expr. Purif. 34(1), 49-55.
38 Coleman, M.A., Lao, V.H., Segelke, B.W. and Beernink, P.T. (2004) J. Proteome Res. 3(5), 1024-1032.
39 Shih, Y.P., Kung, W.M., Chen, J.C., Yeh, C.H., Wang, A.H. and Wang, T.F. (2002) Protein Sci. 11(7), 1714-1719.
40 Hammarstrom, M., Hellgren, N., van Den Berg, S., Berglund, H. and Hard, T. (2002) Protein Sci. 11(2), 313-321.
41 Lesley, S.A., Graziano, J., Cho, C.Y., Knuth, M.W. and Klock, H.E. (2002) Protein Eng. 15(2), 153-160.
42 Waldo, G.S. (2003) Curr. Opin. Chem. Biol. 7(1), 33-38.
43 Millard, C.S., Stols, L., Quartey, P., Kim, Y., Dementieva, I. and Donnelly, M.I. (2003) Protein Expr. Purif. 29(2), 311-320.
44 Prinz, B., Schultchen, J., Rydzewski, R., Holz, C., Boettner, M., Stahl, U. and Lang, C. (2004) J. Struct. Funct. Genomics 5(1-2), 29-44.
45 Stols, L., Millard, C.S., Dementieva, I. and Donnelly, M.I. (2004) J. Struct. Funct. Genomics 5(1-2), 95-102.